National Veterinary Services Laboratories Testing Protocol

Supplemental Assay Method for the Determination of Hydrogen Ion Concentration, Total Nitrogen, TCA Nitrogen, Phenol and Clarity in Intradermic (Filtrate Produced From Cultures of Pn, C, and Dt Strains of *Mycobacterium tuberculosis*) Tuberculin

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1. Introduction

1.1 Background

The Code of Federal Regulations, Title 9 (9 CFR) (Animals and Animal Products) states that the Animal and Plant Health Inspection Service (APHIS) is responsible for administering the Virus-Serum-Toxin Act. It specifies testing methods for licensed tuberculin products. Total nitrogen is determined by classical Kjeldahl digestion, distillation, and titration of the ammonia. Phenol is determined by end-point titration with bromate/bromide. The clarity is determined by visual observation of the solution; it must be clear. Satisfactory pH of the product must be 7.0 ± 0.3. Satisfactory product must contain 0.18 ± 0.06% total nitrogen and 0.047 ± 0.01% TCA-ppt nitrogen. Phenol content must be 0.54 ± 0.04%.

1.2 Key words

tuberculin, nitrogen, intradermic, phenol, pH, TCA nitrogen

2. Materials

2.1 Equipment/instrumentation

2.1.1 pH meter, with combination pH electrode equivalent to Orion 8103 ROSS, capable of measuring from pH 0.000 to 14.000

2.1.2 Balance, top loading, capable of measuring 0.01 g

2.1.3 Digestion unit, Buchi, B-426 with digestion tubes, or equivalent

2.1.4 Distillation unit, Buchi, B-316, or equivalent

2.1.5 Volumetric pipettes, Class A, meet ASTM Standard E969-83

2.1.6 Volumetric flasks, Class A, with barrel head glass stopper, meet ASTM E288 requirements

2.1.7 Erlenmeyer flasks, 125 ml

2.1.8 Buret with PTFE stopcock, 10 ml, precision bore, calibrated to ASTM E-694 accuracy requirements

2.1.9 Buret with PTFE stopcock, 50 ml, precision bore, calibrated to ASTM E-694 requirements

2.1.10 Graduated cylinders [meets ASTM D86, D216, and D447 requirements], 50, 100, 250, 500, and 1,000 ml

2.1.11 Glass-stoppered Erlenmeyer flasks, 250 ml

2.1.12 Heating/stirring plate with stirring bars

2.1.13 Fast filter paper, Whatman No. 1

2.1.14 Disposable beaker, 5 ml

2.1.15 Rubber stopper, No. 1

2.1.16 Small spot light lamp

2.2 Reagents/supplies

All chemicals, reagent grade. Use distilled or demineralized water or water of equivalent purity.

Total and TCA-ppt nitrogen

2.2.1 Sulfuric acid (H_2SO_4)--Purity: Minimum 95.0%, Maximum 98.0%

2.2.2 Mercury Tablets, Brinkmann Instruments, Catalog No. 015-00-646-3

2.2.3 Sodium hydroxide (NaOH)--Purity: 98.5%

2.2.4 Boric acid (H_3BO_3)--Purity: 99.9%

2.2.5 Methyl red--Purity: 98.0%

2.2.6 Hydrochloric acid (HCl)--Assay: 36.5-38.0%

2.2.7 Sodium carbonate (Na_2CO_3)--Purity: 99.9%

2.2.8 Bromo phenol blue--Purity: 98.0%

2.2.9 National Veterinary Services Laboratories (NVSL) Control--Pool of mammalian (intradermic) tuberculin products with established protein and phenol values

2.2.10 Protein-Standard, National Institute of Standards and Technology, Gaithersburg, MD 20899, Standard Reference Material® 927 C, Bovine Serum Albumin, Certified Protein Concentration 71.57 g/L

Phenol (some reagents same as for protein)

2.2.11 Methyl orange--Purity: 98.0%

2.2.12 Silicotungstic acid ($H_4[Si(W_3O_{10})_4]*26H_2O$)--Purity: 99.0% Store at 4°C.

2.2.13 Arsenic trioxide (As_2O_3)--Purity: 99.9%

2.2.14 Sodium bicarbonate ($NaHCO_3$)--Purity: 99.9%

2.2.15 Potassium bromate ($KBrO_3$)--Purity: 98.5%

2.2.16 Potassium bromide (KBr)--Purity: 99.0%

2.2.17 Phenol (C_6H_5OH)--Purity: $\geq$ 99.0%

Hydrogen ion concentration (pH)

2.2.18 Commercial buffers, certified pH 7.00 and pH 4.00

2.2.19 pH electrode storage solution, Orion, Catalog No. 910001, or equivalent

2.2.20 Reference electrode filling solution, Orion, Catalog No. 810007, or equivalent

3. Preparation for the test

3.1 Personnel qualifications/training

No special test-related training is needed for this testing. Analysts performing this procedure should first conduct 2 trial runs using controls and standards and obtain results within acceptable limits.

3.2 Preparation of equipment/instrumentation

3.2.1 pH meter (Calibrate according to the current version of GDOCSOP0009.)

On the Orion 901, dial “Mode” to pH from stand by. Turn “Std Value” wheels to 7.000 and “Slope” wheels to 59.2 (59.2 was selected from the slope vs temperature curve provided by manufacturer). Remove the electrode from the tube of storage solution. Flush the electrode in pH 7 Buffer by means of dipping it in buffer several times, and place it in second cup of fresh buffer.

Wait until the digital millivolt readout becomes stable; hit “Set Concentration” button to 7.00. For pH 4 Buffer, repeat procedure except when the digital readout becomes stable, turn “Slope” wheels until the readout is 4.000. When done, return the electrode to tube of storage solution. Check the level of reference

electrode solution inside of the electrode before and after procedure.

3.2.2 Buchi Kjeldahl equipment

Become familiar with manufacturer's instructions regarding operation. Turn on water that aspirates fumes from suction tube of the digestion unit and keeps the water cool in the condenser of the distillation unit. Adjust water flow in the distillation unit to approximately 1 L per min. Turn on the distillation unit. Set time preselector to "2" (2 min) and stopcock for aspiration to "Off." Make sure that Buchi bottles of NaOH and water are adequate.

3.2.3 For clarity, removal of label from the sealed bottle of tuberculin

When the bottle warms to room temperature (RT), make sure the label is dry and peel off the label from the bottle carefully. Clean the bottle with alcohol and lint-free paper towel.

3.3 Preparation of reagents/control procedures

3.3.1 Protein-Nitrogen test

1. Cut Hg tablets in half.

Caution: Tablets contain mercury; handle in fume hood and wear gloves, safety glasses, and mask.

2. 32% NaOH, dissolve 640 $\pm$ 1 g NaOH in 1.4 L H_2O in 2 L volumetric flask on the magnetic stirrer. Cool to RT. Dilute to volume with H_2O. Repeat above until Buchi 10-L bottle is full. Store at RT.

Caution: NaOH is caustic; avoid contact with skin.

3. Saturated H_3BO_3, add 15 g to 100 ml H_2O. Stir, with heat, until all H_3BO_3 dissolves. Some H_3BO_3 recrystallizes when cool. Store at RT.

4. 0.5% methyl red, dissolve 0.5 g in 100 ml ethanol (95%). Store at 4°C.

5. Standardized 0.01-0.02 N HCl, 1.7 ml HCl/L H_2O. Titrate exactly 0.0100 g dried sodium carbonate dissolved in 25 ml H_2O. Indicator: 3 drops 0.1% bromo phenol blue; the color of endpoint is green, not bluish green nor yellowish green. Store at RT.

6. TCA, dissolve 4 g TCA in 75 ml H_2O in 100-ml volumetric flask. Dilute to volume with H_2O. Store at RT.

7. 0.1% bromo phenol blue, dissolve 0.1 g in 100 ml H_2O. Store at RT.

Calculation:

$$\underline{N}\ HCl = [(g\ Na_2CO_3)x(1000)]/[(Vol\ HCl)\ x\ (52.994)]$$

Caution: Concentrated HCl is corrosive; handle in fume hood. Avoid contact with skin.

8. Nitrogen Standard, dilute protein (**Section 2.2.10**) to the range of 0.16-0.20 protein-N. Prepare sufficient dilution to provide several aliquots of 20-ml portions in 30-ml serum vials. Store at 4°C.

3.3.2 Phenol test (all reagents stable for at least 6 mo unless specified)

1. 20% HCl, slowly add 200 ml HCl to 600 ml H_2O; dilute to 1 L. Store at RT.

2. 0.1% methyl orange, add 0.1 g methyl orange to 100 ml H_2O. Filter if necessary. Store at RT.

3. Silicotungstic acid solution (SAS), dissolve 60 g $H_4[Si(W_3O_{10})_4]*26H_2O$ in 400 ml H_2O in 500-ml volumetric flask. Add 50 ml H_2SO_4. When cool, dilute to volume with H_2O. Store at RT.

4. Clarifying solution (CS), add 50 ml SAS and 125 ml 20% HCl to 325 ml H_2O. Prepare fresh prior to each test.

5. "Acid solution" for As_2O_3 standard solution, add 110 ml HCl and 2.5 ml methyl orange solution to 100 ml H_2O. Store at RT.

6. 0.0500 N As_2O_3, dissolve 2.4730 g dried As_2O_3 in 25 ml hot 1N NaOH in 1-L volumetric flask. Neutralize it with 25 ml 1N H_2SO_4. Cool and dilute to volume with H_2O. Store at RT.

Caution: As_2O_3 is extremely toxic, avoid contact; handle in fume hood using gloves, mask, and goggles. Consult Material Safety Data Sheet for specific handling instructions.

7. Phenol standard, 0.50% dissolve 2.50 g phenol in 500 ml H_2O. Store at RT.

Critical Control Point: The final diluted volume of the test fluid must be adjusted as described in Section 3.3.2.8.

8. Test fluid (TF), dissolve 0.30 g $NaHCO_3$, 1.67 g $KBrO_3$, and 15.00 g KBr in H_2O and qs to 1 L with H_2O. Store at RT. The TF volume must be adjusted by adding corrected volume of H_2O to TF. It must take a volume of 21.3 ml to titrate 25 ml 0.050 N As_2O_3 in 10 ml "Acid Solution." A first time titration will require less than 21.3 ml TF. Adjust as described in the following example:

Example: Assume the first time titration volume is 20.5 ml

(1,000 ml of TF)-(20.5 ml) = 979.5 ml

$$\frac{(979.5)\ (\text{desired vol})}{(\text{actual vol})} \text{ or } \frac{(979.5)\ (21.3)}{(20.5)} = 1{,}017.2 \text{ ml}$$

For corrected volume of H_2O:
1017.2-979.4 = 37.8 ml to be added to TF.

Note: TF in buret has to be put back into flask.

3.4 Preparation of the sample

3.4.1 Receipt

Reference current version of **TCSOP0001**.

3.4.2 Preparation

Licensed or prelicense intradermic tuberculin products are received in sealed serum bottles. They are stored at 4°C in the walk-in refrigerator prior to testing. Before testing, allow sample vials and reagents to warm to room temperature.

4. Performance of the test (use current version of TCFRM0514)

4.1 pH

Flush pH electrode in first cup of 5-ml tuberculin by means of dipping it several times until the pH readout settles, then place electrode in second cup of same fresh tuberculin. Wait until pH readout becomes stable; record pH.

4.1.1 Place 1 ml sample, one-half Hg tablet, and 3 ml H_2SO_4 into a digestion tube

4.2 Clarity

In an area with subdued light, allow your eyes to adjust. Turn on the spotlight lamp, which is positioned upright. Place the unlabeled bottle over the light beam and observe for extraneous particles.

4.3 Total nitrogen

(Analyze the control pool and protein standard each time testing is performed. Analyze each in triplicate.)

4.3.1 Place 1 ml sample, one-half Hg tablet, and 3.0 ± 0.1 ml H_2SO_4 into a digestion tube. Follow the same procedure for the standard and control.

Caution: Hg is poisonous--Use gloves, mask, and goggles.

Caution: Concentrated H_2SO_4 is corrosive--Avoid contact with skin.

4.3.2 Place the tubes in a digestion tube holder. Place the holder into the digestion unit. Turn on the unit and set energy regulator to “5.” Fifteen min later, set to “7.”

4.3.3 Digest until acid comes to true boil or no longer “burned smoke,” about 50-60 min. Set to “9” for 15 more min.

4.3.4 Cool, add 6 ml H_2O, mix, and cool again.

4.3.5 Place digestion tube and a flask containing 5 ml H_3BO_3 and 3 drops indicator into the distillation unit. Tilt the flask so the tip of the condenser is immersed in the H_3BO_3.

4.3.6 Press and hold NaOH button and count to 3. Then hit Start button to start distillation unit. Distill for 2 min.

4.3.7 Titrate collected distillate to endpoint color change of yellow to deep rose (pH 5.0) with standardized HCl. Record the volume of HCl on log sheet.

4.4 TCA-ppt nitrogen

4.4.1 Shake vigorously a 15-ml screw-capped centrifuge tube containing 5 ml sample and 5 ml TCA for 1 min. Let it stand for 10 min.

4.4.2 Centrifuge the tube at 2,500 rpm for 10 min. Discard supernatant.

4.4.3 Add 3 ml H_2O and vortex the tube until the precipitate at bottom mixes. Transfer mix to Kjeldahl flask. Repeat **Section 4.4.3** 2 more times. Then go to **Section 4.3.1** and follow the same procedure as 1 ml sample.

4.5 Phenol

(Analyze the control pool and phenol standards each time testing is performed. Analyze each in triplicate.)

4.5.1 Add 5 ml sample and 100 ml CS to 250-ml glass-stoppered flask. Shake 2 min. Filter through filter paper.

4.5.2 Transfer 50 ml filtrate to another flask. Add 1 drop methyl orange, stopper, and shake a few sec. Observe the color; when red, go to **Section 4.5.3**.

4.5.3 Titrate with 2 ml test fluid (TF), stopper, and shake a few sec. Observe the color. When red, repeat **Section 4.5.3**. When colorless, go to **Section 4.5.4**.

4.5.4 Shake 30 sec. Add 1 drop indicator, stopper, and shake a few sec. Observe the color. When it does not turn to colorless within 10 sec, titrate with 1 ml TF, stopper, and repeat **Section 4.5.4**. When colorless, go to **Section 4.5.5**.

4.5.5 Shake 1 min. Add 1 drop indicator, stopper, and shake a few sec. Observe the color. When red stays longer than 10 sec, titrate with 0.50 ml TF, stopper, and repeat **Section 4.5.5**. When colorless, record total volume of TF as the endpoint of titration and use for calculation of percent phenol.

5. Interpretation of the test results

5.1 pH

No calculation is required.

Satisfactory pH: 7.0 ± 0.3

5.2 Clarity

No calculation is required.

Satisfactory clarity: Negative (no insoluble particles observed)

5.3 Total nitrogen (Report average of triplicates.)

% Total nitrogen = (ml HCl)(N HCl)(1.4008)/(1 ml intradermic)

Satisfactory total nitrogen content: 0.18% ± 0.06%

5.4 TCA-ppt nitrogen

% TCA-ppt nitrogen = (ml HCL)(N HCL)(1.4008)/(5 ml intradermic)

Satisfactory TCA-ppt nitrogen content: 0.047% ± 0.01%

5.5 Phenol (Report average of triplicates.)

% Phenol = (volume of test fluid)(0.04)-(0.04)

Satisfactory Phenol Content: 0.54 ± 0.04%.

5.6 Controls

Results for controls and standards must be within acceptable limits; otherwise repeat testing.

6. Report of test results

Validate and report results according to the current version of **TCSOP0001**.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.406, Revised as of January 01, 2000, page 650.

7.2 Official Methods of Analysis of AOAC International, Arlington, Virginia, 16th Edition, Pat Cuniff, Editor (1995), Volume I, Chapter 12, page 7.

8. Summary of revisions

8.1 Version .01 was written to meet NVSL/CVB Quality Assurance requirements, to clarify practices in use in the NVSL/CVB-L, and to provide additional detail. No significant changes were made from the previous protocol.

8.2 Version .02 was written to clarify practices in use in the NVSL/CVB-L and to provide additional detail. The following are the significant changes made from the superseded protocol:

1. Change in the digestion apparatus

2. Removal of the trichloroacetic acid precipitable protein determination step

8.3 Version .03 was written to clarify practices in use in the NVSL/CVB-L and to provide additional detail. The following is the significant change made from the previous protocol:

1. Correction of cross references in **Section 4**

8.4 Version .04 was written to clarify practices in use in the NVSL/CVB-L and to add the TCA-ppt nitrogen determination step.